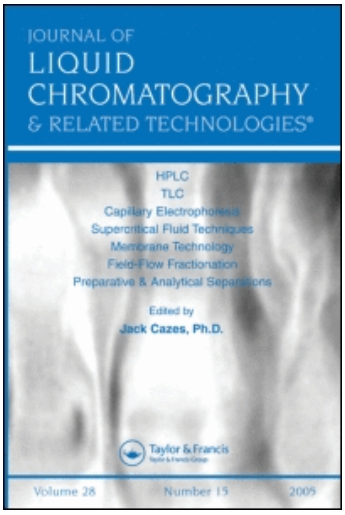

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DETERMINATION OF METRONIDAZOLE IN ADULT *ARTEMIA* USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Sherry Cox,[1] Matthew C. Allender,[2] and Jason Yarbrough[1]
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□ *A new HPLC procedure for the determination of metronidazole, a nitroimidazole, in adult* Artemia *(live brine shrimp) has been developed and validated. Following a liquid extraction using methanol, samples were separated by isocratic reversed-phase HPLC on a Symmetry C_{18} column and quantified using UV detection at 324 nm. The mobile phase was a mixture of water and methanol, with a flow-rate of 1.0 ml/min. The procedure produced a linear curve over the concentration range 1-500 μg/gm with a lower limit of quantification of 0.25 μg/gm. Intra and inter-assay variability was less than 10%. The development of the assay allowed the determination of metronidazole in a live brine shrimp feeding study.*

Keywords antimicrobial, aquaculture, artemia, bioencapsulation, HPLC, metronidazole

INTRODUCTION

The importance of aquaculture has global implications as the world population continues to grow. The success of large scale fish and shrimp farming is commonly jeopardized by the occurrence of infectious diseases, often leading to high mortalities and serious economic losses.[1] In order to manage aquatic animal health the need for antimicrobials is unavoidable. Prophylactic treatment of aquatic animals originally relied on the addition of antibiotics to feed. However, this approach harbors risks for the environment and public health, as excess accumulation of the drug may enter waterways, as well as potentially exposing fish to the toxic effects of the drug. The individual treatment of fish using injections or oral dosing is impractical for management of disease epizootics. However a technique

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of bioencapsulation within adult *Artemia* can be used for the treatment of susceptible infectious diseases in aquatic animals. Bioencapsulation consists of using live feed, supplemented with therapeutic agents.[2,3]

Artemia (live brine shrimp, LBS) is a common food source for a wide range of captive fish. These are highly palatable feed items that are non-selective filter feeders, providing an ideal transport mechanism of soluble pharmaceuticals.[4] Previous studies have shown that *Artemia* can accumulate numerous antimicrobial agents.[5,6] Some of these antimicrobials have been shown to attain therapeutic levels in fish that are fed the *Artemia*.[7] Therefore, standardization of techniques for effectively delivering a wide range of antibiotics to fish through bioencapsulation is needed.

Metronidazole was discovered in 1959 and has been used in human medicine for the treatment of parasitic and bacterial infections. Although there are no veterinary-approved metronidazole products, the drug has been used extensively in the treatment of Giardia in both dogs and cats. It is also used clinically in small animals and horses for the treatment of other parasites and anaerobic infections and could be used in aquatic animals.[8] Currently, metronidazole is not approved for use in fish intended for food, but this method is commonly used for aquarium fish, and its use may be expanded in the future. Metronidazole has been analyzed in plasma[9–14] but few analytical methods have been reported for its determination in tissues. Most of these methods use solid phase extraction techniques[15–17] while one method uses an acetonitrile:perchloric acid solution for extraction.[18] No validated method has been reported for the determination of metronidazole in *Artemia*.

The goal of this work was to develop a simple, specific and reproducible method to determine the concentration of metronidazole in *Artemia* samples. The method has been successfully used in studies at this institution.

EXPERIMENTAL

Reagents and Chemicals

Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Pittsburg, PA). Metronidazole and tinidazole (Figure 1) were purchased from U.S. Pharmacopeia (Rockville, MD). Water was obtained from a Barnstead (Dubuque, IA) Nanopure Infinity ultrapure water system.

Chromatography

The HPLC system consisted of a 2695 separation module and a 2487 UV detector (Waters, Milford, MA). Empower software (Waters) was used

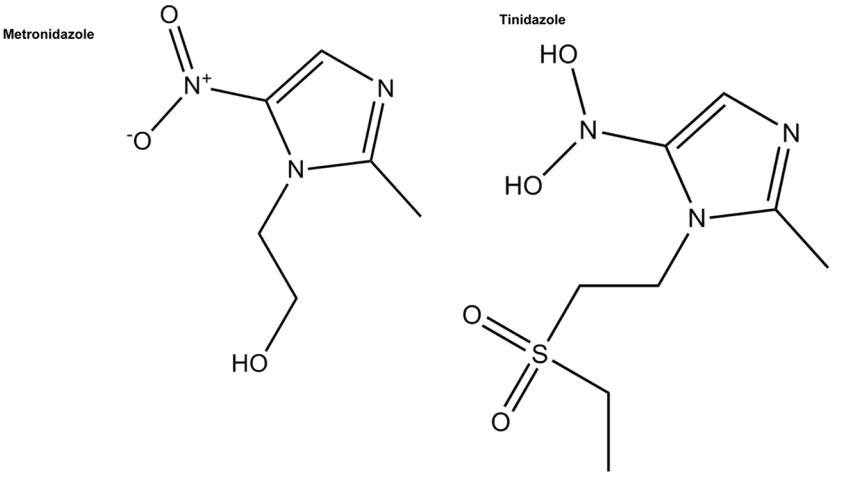

FIGURE 1 Structure of metronidazole and tinidazole.

for data acquisition and processing. Metronidazole was separated on a Waters Symmetry C_{18} (150 mm × 3.9 mm, 5 μm) column with a Symmetry C_{18} (20 mm × 3.9 mm) guard column. The mobile phase was a mixture of water and methanol (90:10 v/v). All solutions were filtered through a 0.22 μm filter and degassed before their use. The water was replaced on a daily basis. The flow rate was 1.0 ml/min and the column temperature was ambient. UV absorbance was measured at 324 nm.

Preparation of Calibration Standards

Metronidazole and tinidazole (internal standard) were dissolved in methanol to produce stock concentrations of 100 μg/gm. Appropriate dilutions of stock concentrations were prepared to produce working stock solutions. Standards were stored at 4°C and were stable for eight months. For preparation of calibration standards and quality control samples, appropriate aliquots of the stock solutions were added to untreated LBS. The final concentrations were 1, 2.5, 5, 10, 25, 50, 100, 300, 400, and 500 μg/gm for the calibration standards and 3, 35, 80 and 200 μg/gm for quality control samples. Calibration standards and control samples were treated the same as samples. Linearity was assessed by linear regression analysis. The calibration curve had to have a correlation coefficient of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except the lower limit of quantification (LLOQ) which was set at 20%.

Sample Preparation

Metronidazole was extracted from LBS using a liquid extraction. Previously frozen LBS were thawed and 50 mg placed into Eppendorf tubes. One hundred and sixty microliters of methanol and 90 µl of the internal standard (tinidazole 100 µg/gm) were added to each tube. Samples were then homogenized using a Power Gen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). The homogenizer was washed in a second Eppendorf tube containing 250 µl of methanol and the solution was transferred to the original sample tube and then centrifuged for 20 minutes at 14000 × g. The supernatant was removed and placed into chromatography vials. A 2.5 µl sample was injected into the HPLC.

RESULTS

Endogenous LBS components did not interfere with the elution of the compounds of interest. Blank LBS samples for specificity testing were prepared in the same way as study samples. Seven different blank LBS samples were used in the pre-validation process and a blank sample from each batch was included in the analysis. Figure 2 shows chromatograms of a (A) blank LBS sample, (B) a 10 µg/gm LBS standard and (C) a LBS sample from a study 4 h after a 2.5 mg/kg dose of metronidazole was administered. Retention times were 6.5 min for metronidazole and 11.0 min for tinidazole.

The tissue peak area ratio (area of metronidazole divided by internal standard area) versus concentration was plotted which produced a linear curve for the concentration range used (1–500 µg/gm) with the correlation coefficients ranging from 0.998 to 0.999. The mean slopes, intercepts and r^2 values are reported in Table 1. Intra-assay RSD for LBS spiked with specific concentrations of metronidazole ranged from 1.4 to 3.4% (Table 1). The LLOQ was 0.25 µg/gm. The inter-assay RSD ranged from 1.9% to 3.5%. The individual values are reported in Table 2. The recovery of metronidazole from spiked LBS was compared with the directly injected analyte at concentrations 3, 35, 80 and 200 µg/gm. Values ranged from 95 to 99% (Table 2). The recovery of the internal standard, tinidazole was 92% at the concentration used in the assay (100 µg/gm). The limit of detection for metronidazole in LBS was 0.1 µg/gm. This represents a peak approximately three times baseline noise. Testing of autosampler and short term stability of standards for 24 hours showed that extractions were stable. The testing revealed that homogenates were stable for 24 hours in an autosampler at ambient temperature. For the concentrations of 3, 35, 80 and 200 µg/gm, there was a 1% drug loss after 24 hours in the autosampler and a 1% drug loss after 24 hours of short

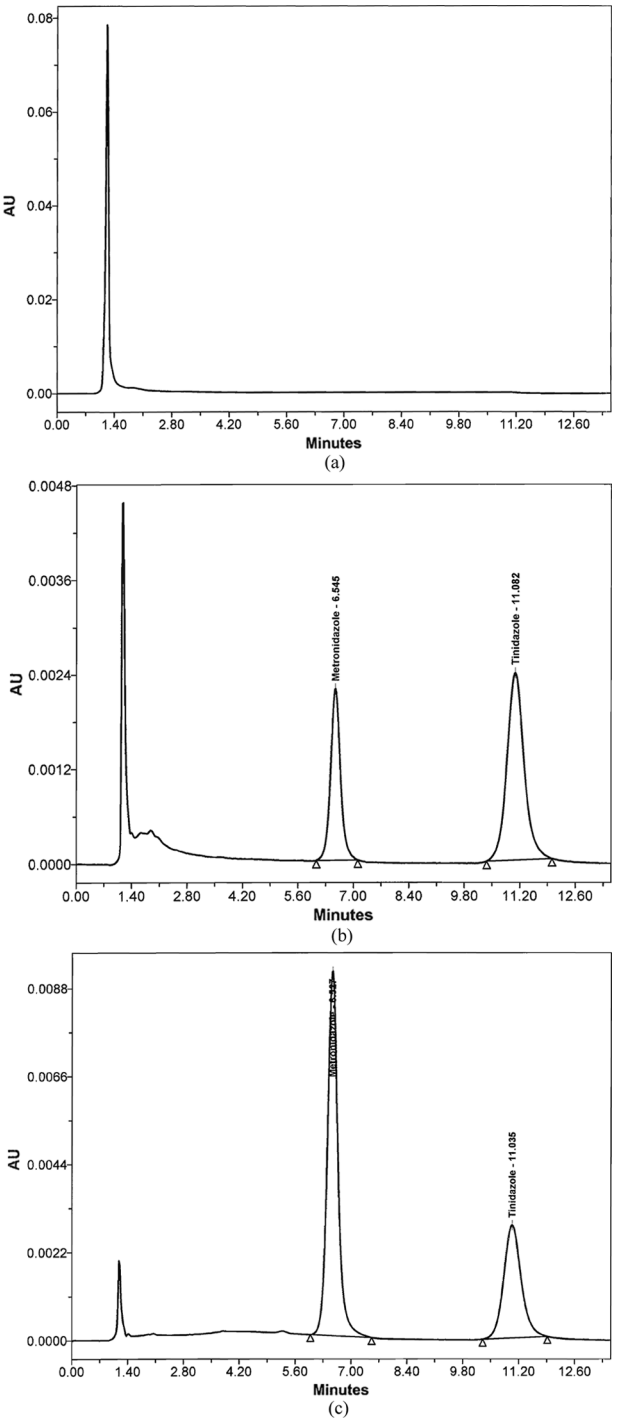

FIGURE 2 Chromatograms for metronidazole. (a) Blank adult *Artemia* sample, (b) 10 μg/gm adult *Artemia* standard (c) and an adult *Artemia* sample after a 2.5 mg/kg dose of metronidazole.

TABLE 1 Intra-Assay Accuracy, Precision and Assay Linearity for Metronidazole in Adult *Artemia*

Concentration Added (μg/gm)	Concentration Measured (μg/gm) (mean ± S.D.)	R.S.D. (%)
Intra-Assay Variability (n = 5)		
3	3.4 ± 0.09	2.9
35	34.1 ± 0.61	1.8
80	81 ± 2.7	3.4
200	199 ± 2.8	1.4
	Mean ± S.D.	R.S.D. (%)
Assay Linearity (n = 5)		
Intercept	−0.04802 ± 0.00245	5.1
Slope	0.04872 ± 0.00475	9.8
r^2	0.9997 ± 0.00023	0.02

S.D.: standard deviation; n: number of samples; RSD: relative standard deviation.

term storage in the refrigerator at 4°C. There was a 2% loss after 2 freeze-thaw cycles.

DISCUSSION

Metronidazole was quantified in LBS by combining a liquid extraction with isocratic reverse-phase HPLC analysis. Several solvents were tested in the extraction process (acetonitrile, methanol, water) as well as Ultrafree-MC filtration units (Millipore). However, methanol produced the largest recovery and optimum peak shape. Stability studies indicate that samples are stable for 24 hours after extraction. The samples can also undergo 2 freeze-thaw cycles with very little drug loss. The mobile phase was a simple combination of water and methanol which required no further manipulation to produce separation of the two compounds.

Most of the methods[16–18] involving the extraction of metronidazole from tissues require the use of solid phase extraction cartridges after

TABLE 2 Inter-Assay Variability and Recovery for Metronidazole in Adult *Artemia* (n = 5)

Concentration Added (μg/gm)	Concentration Measured (μg/gm) (mean ± S.D.)	R.S.D. (%)	Recovery (%)
3	3.4 ± 0.06	1.9	98
35	34 ± 1.2	3.5	95
80	78 ± 2.2	2.8	99
200	200 ± 16.0	8.0	99

SD: standard deviation; n: number of days; RSD: relative standard deviation.

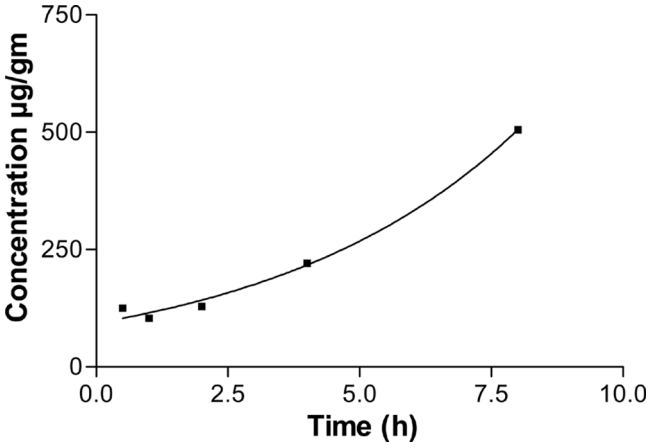

FIGURE 3 Concentration time profile of metronidazole found in *Artemia* after a 2.5 mg/kg dose.

homogenization. These methods also require the use of trichloroacetic acid,[16] a combination of acetonitrile, hexane and sodium sulphate,[17] or a combination of orthophosphoric acid and methanol[15] to homogenize the tissue prior to using the extraction cartridges. The Wibawa et al., method[18] requires that the tissue be snap-frozen in liquid nitrogen, crushed in a table top vice, then re-frozen in liquid nitrogen before the addition of an acetonitrile:perchloric acid mixture for extraction. The supernatant must then be filtered through an Eppendorf filter device before analysis.

Our procedure eliminates the use of solid phase extraction cartridges, hexane, trichloroacetic acid, orthophosphoric acid and the use of Eppendorf filter devices. It is a rugged procedure with the column still in use after 1000 injections. The use of tinidazole as an internal standard corrects for intra- and inter-assay variability in the extraction. The limit of detection and recovery are more than adequate for use in aquatic antimicrobial studies. The injection volume could be increased if a lower limit of quantification is necessary. We did not attempt to shorten the run time because in some samples the endogenous material that eluted at the front of the chromatogram had a slight tail that was longer in some samples than others. The run time was acceptable for our project however, even with the tailing problem the run time could probably be shortened if needed.

CONCLUSION

This analytical procedure was validated in terms of selectivity, recovery, linearity, LLOQ, precision, and accuracy. In conclusion, the results of the study indicate that this HPLC procedure represents a highly specific and

reproducible method that provides consistent quantification of metronidazole in adult Artemia. This method has been used successfully in aquatic studies at this institution (Figure 3).

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